Clin. exp. Immunol. (1980) **41**, 533–540.

The kinetics of T lymphocyte subpopulations in guinea-pigs sensitized with allogeneic transplantation antigens

G. A. HASHIM, N. YEE & W. G. RAMEY *Departments of Surgery and Microbiology, St Luke's-Roosevelt Hospital Center and Columbia University, New York, USA*

(Accepted for publication 21 March 1980)

SUMMARY

The kinetics of specifically sensitized T lymphocytes in the circulation and lymphoid tissues of guinea-pigs immunized with allogeneic transplantation antigens or with synthetic peptide sequence known to induce delayed-type hypersensitivity were documented by the antigen-stimulated active rosette-forming T cell (AgARFC) assay. The results show that immunologically functional cells sensitized to a particular antigen do not remain in the circulation when the antigenic source has been withdrawn. These cells become sequestered in lymphoid tissues and may be recalled into the circulation shortly after the *de novo* administration of sensitizing antigen. The detection of antigen-sensitive T cells in the circulation was indicative of the presence of and failure to detect these cells and their eventual appearance in lymphoid tissues was related to depletion of the antigenic source.

INTRODUCTION

Recent studies from our laboratory showed that a subpopulation of peripheral blood lymphocytes sensitized to specific antigens may be identified by the antigen-stimulated active rosette-forming T cell assay (AgARFC assay) (Hashim, Lee & Pierce, 1977). Significant depression in the number of active but not in the number of total rosette-forming T lymphocytes was demonstrated in peripheral blood of guinea-pigs challenged with encephalitogenic myelin basic protein (BP) or with synthetic peptide antigens known to induce delayed-type hypersensitivity in experimental animals (Hashim *et al.*, 1976). The depression in the active rosette population *in vitro* was reversed by antigenic stimulation *in vivo* (Lee, Hashim & Pierce, 1977). This phenomenon, however, does not suggest depressed cell-mediated immunity but rather a change in lymphocyte surface receptors following *in vivo* cellular sensitization (Hashim *et al.*, 1977; Lee *et al.*, 1977). Further studies from our laboratory showed that the AgARFC assay is useful for detecting cellular sensitivity not only in first-set but also in second-set skin allograft rejection (Ramey *et al.*, 1977). The assay was also informative in monitoring the development of cell-mediated immunity to human histocompatibility antigens in kidney transplantation (Burrows *et al.*, 1978), to myelin BP in multiple sclerosis patients (Hashim *et al.*, 1978) and to tumour-associated antigens in patients with adenocarcinoma of the breast (Ramey *et al.*, 1979a).

The purpose of the present study was to document the kinetics of cell-mediated immunity by the AgARFC assay in blood, lymph nodes and splenic tissues following immunization of guinea-pigs with guinea-pig transplantation antigens or with synthetic peptide known to induce delayed-type hypersensitivity in guinea-pigs. The appearance of antigen-responsive rosette-forming T cells is predicated upon prior immunization with the antigen and the presence of these cells in the circulation is dependent upon the continuous influx of the sensitizing antigen *in vivo*.

Correspondence: Dr G. A. Hashim, St Luke's-Roosevelt Hospital Center, 114th Street and Amsterdam Avenue, New York, NY 10025, USA.

0099-9104/80/0900-0533$02.00 © 1980 Blackwell Scientific Publications

MATERIALS AND METHODS

Animals. Short-hair and Hartley guinea-pigs, weighing 400–500 g, were used throughout this study. The animals were housed in our animal facilities for 1 week before they were studied. They were provided with water and food *ad libitum*. Short-hair and Hartley guinea-pig histocompatibility antigen extracts were prepared from peripheral blood, splenic and lymph node lymphocytes as described in earlier studies (Hashim *et al.*, 1977; Lee *et al.*, 1977). Guinea-pig peripheral blood lymphocytes were isolated from 30 ml heparinized blood by heart puncture under light ether anaesthesia. The blood was diluted 1:1 v/v with Hanks' balanced salt solution (HBSS) (GIBCO, Grand Island, New York) and the lymphocytes were separated by flotation on Ficoll–Hypaque at density 1·08 as described earlier (Hashim *et al.*, 1977). Lymphocytes from splenic tissue and lymph nodes were also dispersed with a pair of tweezers and scissors and eluted through mesh screens with HBSS. The filtered cells were then layered over Ficoll–Hypaque and the lymphocytes were isolated as described earlier.

Short-hair guinea-pig histocompatibility antigen extracts. A lymphocyte suspension of 10^9 cells per 20 ml was prepared and extracted overnight with 3 M potassium chloride as described by Reisfeld, Pellegrino & Kahan (1971) and Meltzer *et al.* (1971). The extract was centrifuged at 105,000 ***g*** for 3 hr and the supernatant dialysed overnight against saline. The residue which formed during dialysis was centrifuged at 10,000 ***g*** and the final supernatant was clarified by centrifugation for 3 hr at 105,000 ***g***. The supernatant was stored at −20°C in small volumes and diluted to desired concentration before use.

Peptide S42. Peptide S42 was prepared in our laboratory by the Merrifield solid-phase peptide synthesis procedure as described earlier (Merrifield, 1963). Peptide S42 is non-encephalitogenic, contains 21 amino acid residues and is known to induce delayed-type hypersensitivity in experimental animals (Hashim *et al.*, 1976). The amino acid sequence of peptide S42 (H-Phe-Ser-Trp-Gln-Lys-Phe-Ser-Trp-Gln-Lys-Phe-Ser-Trp-Gln-Lys-Phe-Ser-Trp-Gln-Lys-OH) is analogous to the encephalitogenic tryptophan region of the myelin BP (Eylar *et al.*, 1971).

Animal sensitization. Each Hartley guinea-pig (400–500 g) received a single injection containing the desired antigen concentration emulsified with an equal volume of Freund's complete adjuvant and 0·1 ml emulsion was injected in one hind footpad. Each animal was sensitized with either 15 μg synthetic peptide S42 or with lymphocyte extract equivalent to $1{\cdot}25 \times 10^7$ cells. The sensitized animals were bled by heart puncture under light ether anaesthesia. Heparinized blood, inguinal lymph node and spleen were isolated under aseptic conditions and then the lymphocytes from each tissue were isolated as described earlier.

Rosette-forming T cell assays. These were performed as described in previous studies (Hashim *et al.*, 1977). Briefly, lymphocytes isolated from blood, lymph node and spleens were assayed separately. The washed lymphocytes were incubated at 37°C for 60 min in the presence of minimum essential medium (MEM) containing 0·05 ml suspension of bacto-latex particles to label phagocytic cells. The incubated cells were then washed three times with HBSS and resuspended with 10% MEM in HBSS at a concentration of about 5×10^6 cells/ml. The total rosette-forming T cell assay (TRFC) was quantitated by the procedure described by Johansen, Johansen & Talmage (1974) for guinea-pig peripheral blood. Approximately 300,000 lymphocytes in 0·05 ml of 10% MEM were mixed with an equal volume of gammaglobulin-free foetal calf serum previously absorbed with rabbit erythrocytes and mixed with 0·05 ml of 2% suspension of RRBC. The mixture was centrifuged at 200 ***g*** for 5 min and then incubated for 60 min at room temperature. The pellet was gently suspended by rolling and rocking motions and a sample was placed in a haemocytometer with a disposable pipette. A minimum of 200 lymphocytes were counted under ×400 magnification, and the percentage of lymphocytes forming rosettes was calculated. A rosette-forming cell was defined as the lymphocyte which showed three or more RRBC adherent to its surface. Latex particle labelled phagocytic cells were excluded from the free lymphocytes. The active rosette-forming T cell assay (ARFC) was determined by a modification of the active rosette technique described for human peripheral blood (Lopez *et al.*, 1974). Lymphocyte suspensions from control and sensitized guinea-pigs (300,000 cells in 0·1 ml of 10% MEM in HBSS) were mixed with 0·05 ml absorbed foetal calf serum and incubated at 37°C for 60 min. To the mixture was added washed RRBC (0·05 ml of 0·25% suspension)

followed by centrifugation at 200 **g** for 5 min. The number of rosette-forming cells was determined as described above.

The antigen-stimulated rosette-forming T cell assay (AgARFC) was performed as follows. Lymphocyte suspension (300,000 cells in 0·05 ml of 10% MEM in HBSS) was mixed with 0·05 ml of the same medium containing $2{\cdot}5\times10^5$ lymphocyte extract equivalent isolated from the peripheral blood of Short-hair guinea-pigs or 1·5 ng peptide S42. The mixture was then incubated at 37°C for 15 min. Following incubation 0·05 ml absorbed foetal calf serum was added and the incubation was continued for an additional 60 min at 37°C. After that, 0·05 ml of 0·25 ml RRBC was added; the mixture was centrifuged at 200 **g** for 5 min and the percentage of rosette-forming cells was determined.

RESULTS

Rosette formation by lymphocytes from normal Hartley guinea-pigs

The percentages of active (ARFC) and total (TRFC) rosette-forming T lymphocytes in peripheral blood, inguinal lymph node and spleen of normal Hartley guinea-pigs are shown in Table 1. The mean per cent total T lymphocytes for peripheral blood was $61{\cdot}2\pm1{\cdot}7\%$ (s.e.m.). Similar values were obtained for inguinal lymph nodes and spleen. The per cent ARFC in peripheral blood was about one-half of the TRFC population. In contrast, higher percentages of ARFC were found in the lymph nodes and spleen. There were no significant increases in the percentages of ARFC or TRFC when normal guinea-pig peripheral blood, inguinal lymph node or splenic lymphocytes were incubated with Short-hair guinea-pig histocompatibility antigen extracts or with synthetic peptide S42. In fact, the data presented in Table 1 show depressed ARFC when inguinal lymph node lymphocytes but not peripheral blood or splenic lymphocytes were incubated with either Short-hair or peptide S42 prior to rosette formation.

Rosette formation by peripheral blood, lymph node and splenic lymphocytes from sensitized Hartley guinea-pigs

Lymphocytes from Hartley guinea-pigs immunized with Short-hair histocompatibility antigen extracts formed higher percentages of rosettes when they were incubated with small amounts of Short-hair antigens (lymphocyte extract equivalent to $2{\cdot}5\times10^5$ cells). Increasing the amount of antigen added to cells *in vitro* did not increase the rosette response any further. Maximum responses were obtained on day 5 following immunization with Short-hair antigen (Fig. 1). Expressed as the

Table 1. Per cent TRFC, ARFC and AgARFC in peripheral blood, lymph nodes and spleen of normal Hartley guinea-pigs

Lymphocyte subpopulation	Peripheral blood	Inguinal lymph nodes	Spleen
TRFC	$61{\cdot}2\pm1{\cdot}4$ (18)	$67{\cdot}3\pm2{\cdot}3$ (6)	$60{\cdot}3\pm0{\cdot}8$ (6)
ARFC	$32{\cdot}8\pm1{\cdot}4$ (9)	$54{\cdot}3\pm3{\cdot}2$ (6)	$41{\cdot}8\pm2{\cdot}7$ (2)
AgARFC (Short-hair)	$35{\cdot}0\pm1{\cdot}9$ (18)	$46{\cdot}2\pm0{\cdot}8$ (6)	$44{\cdot}1\pm2{\cdot}4$ (6)
AgARFC (peptide S42)	$34{\cdot}2\pm2{\cdot}3$ (16)	$46{\cdot}3\pm2{\cdot}6$ (4)	$38{\cdot}8\pm3{\cdot}0$ (4)

Mean per cent ± standard error of total (TRFC), active (ARFC) and antigen-stimulated (AgARFC) rosette-forming T cells in normal Hartley guinea-pigs. The number of samples studied are shown in parentheses incubated in the absence (ARFC) and in the presence (AgARFC) of either Short-hair or peptide S42 antigens.

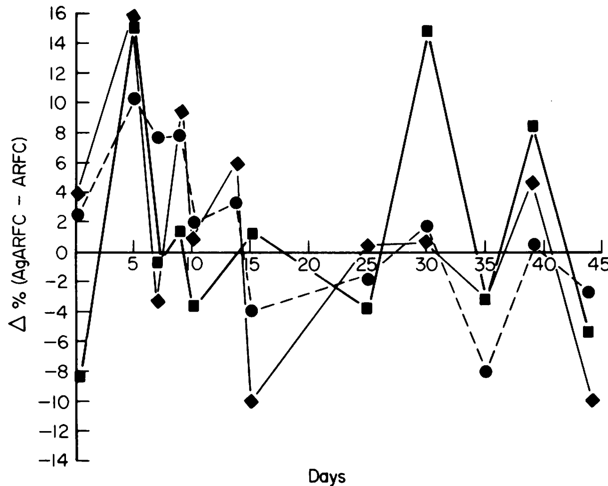

Fig. 1. Hartley guinea-pigs were immunized with Short-hair histocompatibility antigen extracted from peripheral blood lymphocytes. Each guinea-pig received the equivalent of $1 \cdot 25 \times 10^7$ cells extracted to 0·05 ml saline solution emulsified with an equal volume of Freund's complete adjuvant and administered subcutaneously in the hind footpad. On the days indicated following sensitization, the guinea-pigs were bled by heart puncture under light ether anaesthesia, the blood (● - - - ●) collected in heparinized tubes and the inguinal lymph nodes (■—■) and spleens (◆ — ◆) isolated by procedures described in the Materials and Methods section and used for the rosette assays. The viability of isolated lymphocytes was over 95% as determined by the trypan blue dye exclusion method. Each point on the curve represents the mean of three determinations.

difference between the per cent AgARFC and ARFC, the kinetics of the response varied with the tissue from which the lymphocytes were derived. The peak response of peripheral blood lymphocytes was observed on day 5 following challenge and remained significantly high through day 9, dropped to baseline levels between days 10 and 15 and remained there for the duration of the experiment, i.e. 45 days. Similar responses were observed for lymphocytes derived from lymph nodes; however, the peak responses noted on day 5 following immunization dropped to normal

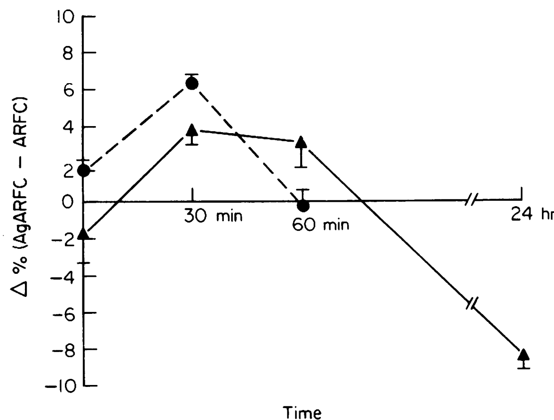

Fig. 2. Hartley guinea-pigs were immunized with either Short-hair guinea-pig histocompatibility antigen extract (● - - - ●) or with peptide S42 (▲—▲). On day 45 post-immunization with Short-hair antigen, the animals were injected subcutaneously with 0·5 ml of a saline solution of the immunizing antigen (equivalent to $1 \cdot 25 \times 10^7$ cell extract). Similarly, on day 30 post-immunization with peptide S42, the animals were injected subcutaneously with 0·1 ml of a saline solution containing 5 μg peptide S42. Following the subcutaneous injections, the immunized guinea-pigs were bled at 30 and 60 min and at 24 hr for the rosette assay.

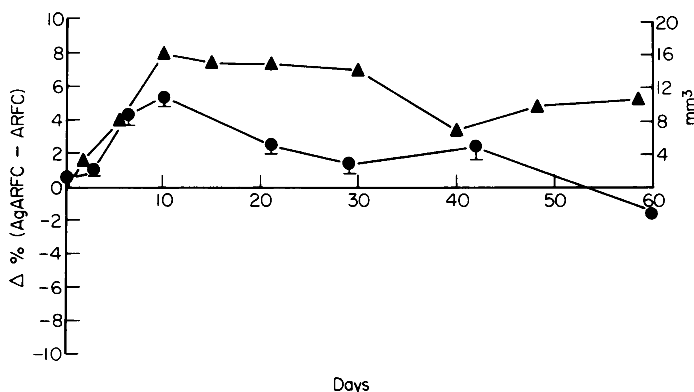

Fig. 3. Delayed-type skin hypersensitivity and antigen-stimulated rosette-forming T cell responses to peptide S42 are shown. Hartley guinea-pigs were immunized with a 15-μg solution of peptide S42 emulsified with an equal volume of Freund's complete adjuvant. A total emulsion of 0·1 ml was injected into the hind footpad of each animal. Twenty-four hours earlier, the immunized animals were skin-tested with a sterile saline solution containing 5 μg peptide S42, and the skin test erythema was measured. The same animals were then bled for the AgARFC assay. Each point on the AgARFC-ARFC curve (●—●) represents the mean ± s.e.m. of triplicate determinations from each of three guinea-pigs. Values for the skin tests (▲—▲) represent the mean values (averages of two cross-sectional diameters in mm) of the erythematous areas measured in the same three guinea-pigs.

levels by day 7 and remained at baseline until day 25. Between days 25 and 45, two additional peaks were observed which occurred on days 30 and 39 (Fig. 1). The kinetics of the response observed for splenic lymphocytes showed three separate peak responses on days 5, 9 and 14 respectively (Fig. 1). The peak response of a 16% rise in AgARFC-ARFC differences which occurred on day 5, dropped to 9 and 6% on days 9 and 14 respectively. The AgARFC cells remained at baseline levels until day 39, when a second response was observed which decayed to below baseline within 3 days. Similarly, higher percentages of rosette-forming T cells were obtained when peripheral blood lymphocytes from Hartley guinea-pigs sensitized with peptide S42 were preincubated with the sensitizing antigen. The initial peak of antigen-responsive lymphocytes which developed between days 3 and 5 gradually decayed to normal by day 14. From days 15 to 40, the percentages of AgARFC-ARFC were below normal levels; however, by day 60, peptide S42-responsive cells rose above the baseline.

Effect of antigenic boosters upon rosette formation in sensitized guinea-pigs

Hartley guinea-pigs sensitized with a single injection of either Short-hair or peptide S42 antigens were bled for rosettes on days 45 and 30 following sensitization, respectively. At this time peripheral blood lymphocytes failed to respond to antigenic stimulation by the AgARFC assay (Fig. 2); however, the administration of saline solutions of either Short-hair antigen extract or peptide S42 subcutaneously to respectively sensitized animals brought about positive AgARFC responses within 30 min. These responses were maintained for 30 to 60 min following the administration of the antigen into the skin; then decayed to original or below original baseline values (Fig. 2).

Correlation between delayed-type skin hypersensitivity and AgARFC responses in sensitized guinea-pigs

Simultaneous measurements of delayed-type skin hypersensitivity and AgARFC responses in peptide S42-sensitized animals are shown in Fig. 3. The animals were bled for the AgARFC assay 24 hr after administration of the skin test antigen. At this time the erythema with induration and sometimes with central necrosis reached peak values. Peak responses for both the skin tests and the AgARFC assays occurred 10 days following immunization. Daily injections of 5 μg peptide S42 into the skin 24 hr before the AgARFC assay elicited positive skin tests as well as AgARFC

responses. These responses were maintained for 50 to 60 days as long as the animals received daily antigenic boosters.

DISCUSSION

Studies of rosette formation in experimental animals have largely focused upon evaluating the total number of T cells in peripheral blood and in a variety of lymphoid tissues (Gupta & Grieco, 1974; Johansen *et al.*, 1974; Scheper *et al.*, 1975; Siegel, 1970). Rosette formation between guinea-pig lymphocytes and rabbit red blood cells is considered a marker for guinea-pig thymus-derived lymphocytes (Lopez *et al.*, 1974; Revell, Wilson & Coombs, 1974; Stadecker, Bishop & Wortis, 1973; Wilson & Coombs, 1973) and analogous to the specific binding of sheep red blood cells to human thymocytes (Jondal, Holm & Wigzell, 1972; Scheper *et al.*, 1975; Stadecker *et al.*, 1973).

Our results show the presence of three subpopulations of rosette-forming T cells not only in the peripheral blood but also in lymph nodes and spleens of immunized animals. The three subpopulations of T lymphocytes have been designated as total (TRFC), active (ARFC) and antigen-stimulated (AgARFC) rosette-forming T cells. The presence of AgARFC subpopulations in either of the three tissues studied is predicated upon prior immunization of the respective animal. The per cent TRFC in normal Hartley guinea-pig peripheral blood was about 60%, and similar values were obtained for TRFC of inguinal lymph node and splenic lymphocytes. These values are in agreement with those previously reported for spleen and peripheral blood T lymphocytes for Hartley guinea-pigs (Hashim *et al.*, 1977; Johansen *et al.*, 1974) and parallel the total T lymphocyte population reported for human peripheral blood (Gupta & Grieco, 1974; Hashim *et al.*, 1978; Yu, 1975). The per cent ARFC in the peripheral blood of normal Hartley guinea-pigs was approximately 50% of the TRFC subpopulation. In contrast, the per cent ARFC for inguinal lymph node and splenic lymphocytes was significantly higher; e.g. approximately 87 and 78% of the TRFC population of the respective tissues.

The per cent TRFC and ARFC were not increased following incubation of lymphocytes from normal animals with antigens before rosette formation. These results suggest that the antigen-induced increase in rosette formation is related to the status of cellular sensitization of a particular animal. Normal lymphocyte rosette-unresponsiveness to antigenic stimulation *in vitro* paralleled the unresponsiveness of respective animals to the same antigen when the latter failed to elicit delayed-type skin hypersensitivity in normal animals *in vivo* (Lee *et al.*, 1977). It may be concluded that the AgARFC assay is a specific measure of cell-mediated immunity that can be demonstrated only with specific antigens used for sensitization. This conclusion is supported by the finding that a positive AgARFC assay was obtained in guinea-pigs sensitized with either Short-hair histocompatibility antigen extracts or with peptide S42. Both antigens are known to induce cell-mediated immunity that can be elicited by only the sensitizing antigen (Hashim *et al.*, 1976; Ramey *et al.*, 1977). Demonstrated by the AgARFC assay, cell-mediated immunity to single injections of small amounts of either Short-hair or peptide S42 antigens persisted in the peripheral blood for more than 10 days and virtually disappeared from the circulation. Similar trends were observed in the draining of inguinal lymph node and splenic lymphocytes of sensitized animals. The kinetics of the AgARFC response in terms of responsiveness to antigenic stimulation *in vitro* for either Short-hair or peptide S42 antigens was the same in respectively sensitized animals despite differences in physico-chemical as well as biological properties of both antigens.

The kinetics of cell-mediated immune responses to either antigen were demonstrated as early as 3 days following immunization and their subsequent decay between days 10 and 15 did not appear to be influenced by the presence or absence of humoral immunity. In the Short-hair-immunized animals, cytotoxic antibodies specific for Short-hair histocompatibility antigen have been detected in the serum of immunized guinea-pigs (Ramey & Hashim, unpublished observations); however, the administration of peptide S42 has not been shown to induce the formation of humoral immunity in experimental animals (Hashim *et al.*, 1976).

Peptide S42-sensitized T cells were demonstrated by delayed-type skin hypersensitivity within 3 to 4 days following single injections of peptide S42 (Hashim *et al.*, 1976). Our results further show

that the subpopulation of antigen-sensitive T cells eventually disappears from the circulation. Longitudinal studies of peripheral blood, lymph node and splenic lymphocytes from sensitized animals revealed that the disappearance from the circulation of antigen-sensitive T cells is followed by their eventual appearance in lymph node and in splenic tissues. These cells may be recalled into the circulation within 30 min following the *de novo* administration of small amounts of the sensitizing antigen into the skin suggesting that the presence of antigen-sensitive T cells or their recall from the lymphoid tissues into the circulation is directly related to the availability of an antigenic source. This observation is supported by the finding that the continuous presence of antigen-sensitive T cells in the circulation may be maintained by periodic administration of small amounts of antigen. It may be concluded that once the antigen was processed and its source has been depleted, the sensitized T cells disappeared from the circulation and became sequestered into the lymphoid tissue. Further experimental support for this conclusion was demonstrated *in vivo* by the results of the skin test assays. The kinetics of the delayed-type skin hypersensitivity responses paralleled those obtained by the AgARFC assays done in the same animals. The short-term recall of antigen-sensitive T cells into the circulation by a single antigenic boost is effectively matched by their long-term presence following periodic antigenic boost. These results suggest that the absence from the circulation of T lymphocytes sensitized to a particular donor antigen does not rule out their presence in other tissues. The presence or absence of these lymphocytes from a particular recipient may be documented by the skin test or the AgARFC assays shortly after the subcutaneous administration of a very small amount (in sterile saline) of the sensitizing or donor antigens.

The kinetics of antigen-sensitive peripheral blood T lymphocytes are important factors in diagnosis and therapy of cell-mediated immune disorders. The percentage of absolute numbers of circulating T lymphocytes sensitized to a particular antigen need not fall within the 0·1 to 3·0% clonal limits. Depending upon the time at which they are measured, the percentage of antigen-sensitive T lymphocytes may be as high as 16% or higher. The peak response observed following a single antigenic challenge is followed by gradual decay and eventual disappearance of sensitized lymphocytes from the blood into the lymphoid tissue. Our results further suggest that changes in levels of circulating antigen-sensitive T lymphocytes are important factors in evaluating the immunological status of patients with primary and metastatic neoplasia (Ramey *et al.*, 1979a, b), in human kidney transplantation (Burrows *et al.*, 1978) and in autoimmune diseases such as multiple sclerosis (Hashim *et al.*, 1978).

A portion of this work was in partial fulfilment of the requirements for a MS degree from Long Island University, Brooklyn, New York by N. Yee.

This study was supported by a grant from The Clark Foundation, the National Multiple Sclerosis Society, grant No. RG 1197-A-4, and a grant from The Margaret T. Biddle Foundation.

REFERENCES

Burrows, W.B., Pierce, J.C., Hashim, G.A., Ramey, W.G., Swistel, A.J. & Fitzpatrick, H.F. (1978) Cell-mediated immunity to donor antigens in renal allograft recipients. *Surgery*, **83**, 741.

Eylar, E.H., Brostoff, S., Hashim, G.A., Caccam, J. & Burnett, P. (1971) Basic A1 protein of the myelin membrane: the complete amino acid sequence. *J. biol. Chem.* **246**, 5770.

Gupta, S. & Grieco, M.H. (1974) E Rosette test. *Lancet*, **ii**, 954.

Hashim, G.A., Lee, D.H. & Pierce, J.C. (1977) Antigen stimulated rosette formation by T lymphocyte in experimental allergic encephalomyelitis. *Neurochem. Res.* **2**, 99.

Hashim, G.A., Lee, D.H., Pierce, J.C., Braun, C.W. & Fitzpatrick, H.F. (1978) Myelin basic protein stimulated rosette-forming T cells in multiple sclerosis. *Neurochem. Res.* **3**, 37.

Hashim, G.A., Sharpe, R.D., Carvalho, E.F. & Stevens, L.E. (1976) Suppression and reversal of allergic encephalomyelitis in guinea pigs with a non-encephalitogenic analogue of the tryptophan region of the myelin basic protein. *J. Immunol.* **116**, 126.

Johansen, K.S., Johansen, T.S. & Talmage, D.W. (1974) T cell rosette formation in primates, pigs and guinea pigs. *J. Allergy clin. Immunol.* **54**, 86.

Jondall, M., Holm, G. & Wigzell, H. (1972) Surface markers on human T and B lymphocytes. *J. exp. Med.* **136**, 207.

Lee, D.H., Hashim, G.A. & Pierce, J.C. (1977) Correlation between antigen induced rosette formation and cutaneous delayed hypersensitivity in experimental allergic encephalomyelitis. *Neurochem. Res.* **2**, 293.

Lopez, L.R., Johansen, K.S., Radovich, J. & Tal-

MAGE, D.W. (1974) Thymocyte, macrophages and erythrocytes. *J. Allergy clin. Immunol.* **53,** 336.

MELTZER, M.S., LEONARD, E.J., RAPP, H.J. & BORSOS, T. (1971) Tumor-specific antigen solubilized by hypertonic potassium chloride. *J. Natl. Cancer Inst.* **47,** 703.

MERRIFIELD, R.B. (1963) Solidphase peptide synthesis. *J. Am. Chem. Soc.* **85,** 2149.

RAMEY, W.G., HASHIM, G.A., BURROWS, W.B., SWISTEL, A.J., MUNTHER, A. & FITZPATRICK, H.F. (1979a) Detection of breast tumor antigen-sensitive circulating T lymphocytes by antigen stimulated active rosette formation. *Cancer Res.* **39,** 4796.

RAMEY, W.G., HASHIM, G.A. & FITZPATRICK, H.F. (1979b) Circulating breast tumor antigen-sensitive T lymphocytes in early breast cancer and high risk benign breast disease. *Br. J. Surg.* **66,** 848.

RAMEY, W.G., HASHIM, G.A., PIERCE, J.C. & FITZPATRICK, H.F. (1977) A new assay for T cell-mediated immunity to transplantation antigens. *Surgery,* **81,** 640.

REISFELD, R.A.,PELLEGRINO, M.A. & KAHAN, B.D. (1971) Salt extraction of soluble HLA antigens. *Science,* **172,** 1134.

REVELL, P.A., WILSON, A.B. & COOMBS, R.R.A. (1974) Rosette formation of guinea pig lymphocytes and killer cells characteristics by rosette forming reaction. *Int. Arch. Allergy,* **47,** 703.

SCHEPER, R.J., BAAK, J.P.A., HISIVARA, S. & VELDHUIZEN, R.W. (1975) RBC and EAE rosette formation after DNCB contact sensitization and glycophosphamide treatment of guinea pigs. *Immunology,* **29,** 909.

SIEGEL, I. (1970) Nature and antibody induced adherence of guinea pig phagocytic cells to autologous and heterologous thymocytes. *J. Immunol.* **105,** 879.

STADECKER, M.L., BISHOP, G. & WORTIS, H.H. (1973) Rosette formation by guinea pig thymocytes and thymus derived lymphocytes with rabbit red blood cell. *J. Immunol.* **111,** 1834.

WILSON, A.B. & COOMBS, R.R.A. (1973) Rosette formation between guinea pig lymphoid cells and rabbit erythrocytes, a possible T-cell marker. *Intl. Arch. Allergy appl. Immunol.* **44,** 544.

YU, D.T.Y. (1975) Human lymphocyte subpopulation: early and late rosettes. *J. Immunol.* **115,** 91.